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RESEARCH ARTICLE

Behavioural evidence for a sleep-like quiescent state in a pulmonate mollusc, *Lymnaea stagnalis* (Linnaeus)

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SUMMARY

The objective of this study was to determine whether the great pond snail, *Lymnaea stagnalis*, expresses a sleep-like behavioural state. We found that snails spontaneously enter a relatively brief (22±1 min) quiescent state characterized by postural relaxation of the foot, mantle and tentacles, and cessation of radula rasping. Quiescence was reversed ('aroused') by appetitive (sucrose solution) and aversive (tactile) stimuli. Responsiveness to both stimuli was significantly lower in quiescent snails than in active snails. However, tactile stimuli evoked a more sustained defensive response in quiescent snails. Quiescence bouts were consolidated into 'clusters' over an infradian timescale and were only weakly affected by time of day. Clusters contained 7±0.5 bouts, lasted 13±1 h and were separated by long (37±4 h) intervals of almost continuous activity. Analysis of Kaplan–Meier survival curves revealed that the quiescent bout duration was described by an exponential probability distribution (time constant 15±1 min). Active bout duration was described by a bi-exponential probability distribution (time constants 62±4 and 592±48 min). We found no evidence for a 'sleep rebound' mechanism and quiescence expression appeared to be regulated through stochastic processes causing state transitions to resemble a Markovian random walk. We conclude that *Lymnaea* is a potentially valuable model system for studies of cellular function in sleep.

Key words: sleep, mollusc, model organism, *Lymnaea stagnalis*.

INTRODUCTION

The study of sleep is growing rapidly, as is our awareness of the vital importance of this behavioural state to basic biology and clinical medicine. Yet some of the most fundamental questions about sleep remain unanswered (Franken et al., 2009), including what are its functions, how much is needed and how did it evolve? In recent years, progress has been made in the areas of sleep-related aspects of molecular biology (Tafti and Franken, 2007) and the roles of sleep in learning and memory (Diekelmann and Born, 2010; Graves et al., 2001). Sleep-related molecular mechanisms have been studied in several animal model systems, and research using the genetically tractable fruit fly (*Drosophila melanogaster*) has proven very productive (Hendricks and Sehgal, 2004; Shaw, 2003). Such successes highlight the advantages of studying sleep in a simple animal model (Hendricks et al., 2000b). Studies of sleep-like behaviour in invertebrates, and comparisons of similarities and differences between vertebrates and invertebrates, may yield important insights into the evolutionary origin of sleep (Piscopo, 2009). Unfortunately, our knowledge of invertebrate sleep biology lags far behind that of vertebrates, despite the recent surge of sleep studies in *Drosophila*.

It is widely hypothesized that sleep has a functional role in learning and memory (Diekelmann and Born, 2010), although the issue remains controversial (Vertes and Siegel, 2005). *Drosophila* has proven very useful in the study of both general and sleep-related molecular aspects of learning and memory (Graves et al., 2001), but flies present significant practical disadvantages for studies in synaptic plasticity and there remains a need for a neurophysiologically tractable animal model of sleep and memory. Many of the fundamental mechanisms of neuronal plasticity underlying memory formation were discovered using molluscan preparations (Kandel, 2001), which present many practical advantages to the neurophysiologist (Mills and Winlow, 1979; Lukowiak et al., 2003). The sea hare (*Aplysia californica*) and the great pond snail (*Lymnaea stagnalis*) are now well established as animal models of learning and memory (Kandel, 2001; Lukowiak et al., 2006). Recent studies have demonstrated an influence of the circadian timing system in learning and memory formation in these species (Fernandez et al., 2003; Wagatsuma et al., 2004), but they have not been well studied in the context of sleep. Indeed, it has not yet been determined whether these molluscs do in fact sleep. Studies in *Octopus* (Brown et al., 2006; Cobb et al., 1995) and anecdotal observations in *Aplysia* (Strumwasser, 1971; Susswein et al., 1983; Ziv et al., 1991) represent, to our knowledge, the only evidence that molluscs express a sleep-like resting state.

There is no single characteristic that unequivocally defines the sleeping state; instead several criteria are used collectively to discriminate sleep from other behavioural states (Hendricks et al., 2000b). The key criteria used to define behavioural sleep are quiescence and reduced responsiveness to sensory stimuli, both of which must be rapidly reversible, either spontaneously or in response to a moderately strong natural stimulus. Quiescence is not specific to sleep; resting wakefulness, paralysis, hibernation, torpor, akinesis, diapause, aestivation, anaesthesia and coma all share this characteristic. However, only in sleep and resting wakefulness is the suppression of motor output rapidly and spontaneously reversible. The second criterion, reversible suppression of sensory responsiveness, characterizes a quiescent state as sleep-like, and

distinguishes it from vigilant rest. In human beings, the reversible suppression of sensory and motor function is accompanied by a reversible loss of conscious awareness but the latter is not amenable to direct measurement in other animal species. Neurophysiological correlates of sleeping behaviour are well defined in mammals (Dijk, 1995). However, although a few notable studies have reported state-dependent neuronal activity in several different invertebrate species (Kaiser and Steiner-Kaiser, 1983; Nitz et al., 2002; Ramon et al., 2004; Brown et al., 2006), a consistent pattern of electrophysiological correlates of sleep-like behaviour has not yet been established in invertebrates. Thus, the designation of a resting state as sleep-like is presently based only on behavioural criteria in invertebrates. Additional defining criteria include stereotypical species-specific posture and sleeping location, and 'homeostatic' and circadian regulation. These latter characteristics are indirectly supportive of a claim that a quiescent behaviour is sleep-like, but they are neither necessary nor sufficient to unequivocally define the sleeping state.

The objective of the present study was to evaluate whether *L. stagnalis* exhibits the behavioural characteristics of a sleep-like state, and thereby to determine whether this organism is potentially useful as a simple animal model in sleep research.

MATERIALS AND METHODS

Animal source and housing

Lymnaea stagnalis are found throughout Europe and Northern Asia in slowly moving or stagnant freshwater ponds, rivers and lakes. They are hermaphroditic, omnivorous and are bimodal breathers, using both the skin and a simple lung (the pneumostome) for respiratory gas exchange. The animals used in the present study were generously provided by Dr J.-P. Feng from her laboratory colony at the University of Toronto, Toronto, Canada. This strain was originally obtained from canals in The Netherlands in the early 1950s and has been maintained in captivity over many generations since then. Stock animals were maintained in our laboratory in an aerated 20 l freshwater aquarium at 23°C under a natural light-dark cycle (i.e. on a window sill) until used for experiments. They were fed *ad libitum* lettuce leaf, small pieces of carrot and commercial aquarium algae wafers (Hikari Sales USA Inc., Hayward, CA, USA). Adult animals (>1.5 cm shell length, >2 months old) were used in all experiments.

Three series of studies were undertaken. (1) A preliminary observation of the behaviour of animals in the stock tank, the purpose of which was to identify candidate behaviours that could be evaluated as potential sleep-like states. (2) A total of 306 snails were used in tests of response sensitivity to sensory stimulation. In all cases, each animal was used only once (i.e. for a single trial of a single test). (3) Eight additional snails were used in a detailed analysis of quiescence behaviour using time-lapse video recordings over 79 days, including variations in lighting schedule (see below).

Behavioural categories

Direct visual observation of animals in the stock tank led to the designation of six categories of behaviour: locomotion, feeding, defensive withdrawal, oviposition, aerial respiration and quiescence. The quiescent behaviour was evaluated as a candidate sleep-like behaviour and, except where noted, all other behaviours were collectively classed as 'active'. A detailed analysis of the full ethogram of this species was beyond the scope of the present study.

(1) Snail behaviour was classified as 'locomotion' when moving on a solid surface or across the surface of the water. During locomotion the foot was extended longitudinally with an aspect ratio (length/width) of approximately 2.1–2.5 (Fig. 1B).

(2) Behaviour was classified as 'feeding' during intermittent pauses in locomotion with continued rasping movements of the radula. The foot remained extended during these feeding pauses.

(3) 'Defensive withdrawal' occurred in response to tactile stimulation and was transient, lasting less than 1 min in most cases. This behaviour was variable in extent, depending upon the stimulus intensity. Under the conditions of this study (see below), the response was incomplete, and involved retraction of the tentacles, head and anterior part of the foot into the shell and a reduction of the foot aspect ratio to approximately 1.5–2.0 (Fig. 1C). Following this initial withdrawal response the snails usually re-emerged, changed direction and moved away from the site of stimulation. This behaviour was never observed to occur spontaneously.

(4) 'Oviposition' included periods of activity and inactivity. During the inactive phase the foot was often extended longitudinally and curved. Active periods involved circular movements and brief lateral movements of the shell. The latter were very distinctive during high-speed review of time-lapse video recordings and made it possible to distinguish pauses in oviposition from quiescence.

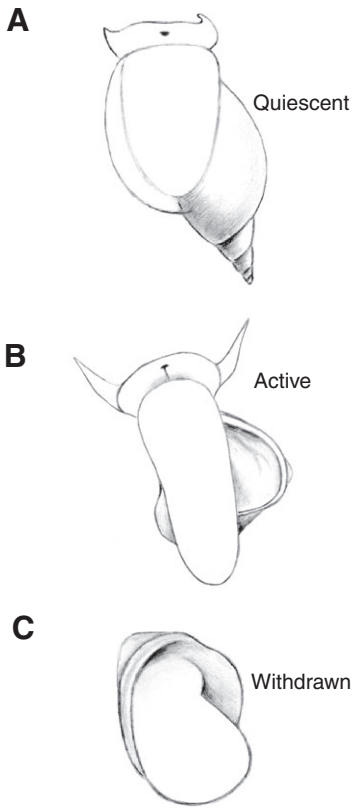

Fig. 1. Line drawings of snails exhibiting the three main categories of behaviour measured in this study. (A) Quiescence: featuring immobility, partial relaxation of the foot, head and tentacles, absence of radula rasping and a passive partial 'sinking' of the shell. Snails remained attached to the substrate and only partially protected by the shell. (B) Active locomotion: showing elongated foot and tentacles, and an extended head. Reorientation of the shell and radula activity were also commonly observed in active snails. (C) Defensive withdrawal: observed only following experimental application of tactile stimuli to the head. Note that this is an incomplete withdrawal, mainly involving the anterior part of the animal, with retraction of the tentacles and head into the shell.

(5) Snails returned to the water surface at irregular intervals to breathe. 'Aerial respiration' included a period of immobility with one or more cycles of opening and closing of the pneumostome. Depending upon the orientation of the snail relative to the camera, it was not always possible to identify respiration when snails were immobile at the water surface in time-lapse recordings. The water in the stock tank and observation tank was fully aerated to reduce aerial respiration.

(6) 'Quiescent' snails were attached to a solid surface in what appeared to be a relaxed immobile posture, the foot was bilaterally symmetrical (i.e. not curved as in oviposition) but less extended than when active or during feeding pauses (i.e. quiescent aspect ratio was approximately 1.7–2.0; Fig. 1A). In contrast to feeding, there was an absence of radular movement during quiescence. Quiescent snails exhibited partially extended tentacles, and when attached to vertical surfaces the shell appeared to hang away from the foot slightly, i.e. the shell was not pulled forward as it is during defensive withdrawal. As respiration was not always easy to detect on video (see 5, above), quiescence was separately recorded as 'submerged quiescence' and 'surface quiescence'. The latter was defined as a snail attached to the substrate within 2 cm of the water surface.

Stimulus response sensitivity

The responsiveness of snails to appetitive and aversive stimuli were compared between submerged quiescence and locomotion. These tests were performed in the stock tank to avoid disturbing the snails prior to testing. In all tests, each snail was subjected to a single trial and then removed from the tank. For trials during quiescence, snails were quiescent for >5 min before application of the stimulus. For trials during locomotion, snails were tested when travelling in a lateral direction and fully adhered to the substrate; those in the process of changing direction, interacting with conspecifics, or with a section of the foot not attached to substrate, were ignored.

The appetitive stimulus consisted of 0.4 ml of a 0.1 mmol l^{-1} solution of sucrose in distilled water applied as a bolus to the dorsal surface of the head via a 1 ml syringe. Responses were recorded as latency to radula bite response and subsequent bite rate (bites per 30 s). These two variables were recorded in separate trials, one trial per animal, in a total of 138 snails. Latency to bite was defined as the period of time between treatment contact and the end of the first full bite response. If a snail did not respond within one minute, it was recorded as a 'No Response' and removed from the tank. A 30 s cut-off was selected for bite rate measurements because bite rate was observed to decrease after approximately that time. To control for any visual or mechanical disturbance caused by the application of the solution, a separate group of 84 snails were also stimulated in an identical manner but using water instead of sucrose solution (sham appetitive stimuli).

Aversive response sensitivity was assessed by observing withdrawal responses to tactile stimuli in 42 snails. A custom-made probe was constructed and calibrated to administer a 0.004–0.008 N force. The probe consisted of a spring-loaded stainless steel rod with a rounded tip (diameter = 3 mm) and this was applied by hand in a 'poking' motion with the probe held perpendicular to the dorsal surface of the head. If no withdrawal response was elicited within 15 s of probe application another stimulus was applied and this was repeated until a withdrawal response occurred. Responsiveness was estimated as the number of stimuli required to elicit a withdrawal response. Response duration was assumed to be indicative of response intensity, and was recorded as the time to post-withdrawal re-emergence (i.e. resumption of pre-stimulus posture) following the stimulus. To control for any non-specific effects of probe placement, the above protocol was repeated in 42 snails, but without the probe making contact with the snail (sham aversive stimuli).

Temporal organization of quiescence

Eight adult snails were observed in an aerated aquarium over a 79 day period using time-lapse video (model CCD-TRV228, Sony Canada, Toronto, ON, Canada). Each snail was placed in a separate vertical observation channel constructed using white Plexiglas® dividers placed against the glass front wall of the tank. Small spaces in the dividers allowed circulation of water within and between channels. Each channel was 25 cm high (water surface to bottom), 4 cm wide and 5 cm deep (glass front to back walls). The divider walls were angled appropriately to eliminate blind spots when the camera was positioned 1 m in front-centre of the tank. Data were recorded at a rate of 4 frames min^{-1} in standard MPEG format. A digital time stamp was recorded in each frame and used as reference during subsequent playback and analysis.

The light:dark (LD) cycle varied as follows: days 1–21, 16 h:8 h LD (lights on 05:00 h, off 21:00 h; hereafter termed LD16:8); days 22–31, DD (continuous darkness); days 32–48, LD16:8; days 49–68, LL (continuous light); days 69–79, LD16:8. During LL and the light phase of LD, light intensity was approximately 110 lux at the water surface. Throughout the study the observation tank was illuminated by two infra-red lights, which enabled recording in the dark. A long photoperiod was used in LD for two reasons. (1) Data were recorded in late autumn and early winter when snails have an increased propensity to hibernate (Copping et al., 2000). We used simulated summer photoperiod to reduce the likelihood of this, and indeed did not observe any hibernating snails in this time-lapse study. (2) Visual scoring of the time-lapse images was more difficult and time-consuming for recordings made in darkness, a problem that was minimized with short nights. Owing to technical problems, data were not recorded on 4 days in LD and 2 days in LL. Hence, the complete dataset comprised 45 days in LD, 10 days in DD and 18 days in LL.

Durations of quiescent bouts and time of bout onset were recorded, together with the corresponding inter-bout intervals. Surface quiescent bouts and submerged quiescent bouts were compared using paired *t*-test. For each variable, the data were binned in 4 h daily intervals for analysis of daily rhythms. Repeated measures ANOVA was used to test for differences across the six intervals of the day using data recorded under LD16:8 (i.e. the lighting conditions under which any circadian rhythms would be entrained to a 24 h period and synchronized across animals). In LD, data were also pooled into 'light' [intervals 1–4, Zeitgeber time (ZT) 0–16 h] and 'dark' (intervals 5 and 6, ZT16–24 h) and compared using paired *t*-test. The effect of lighting conditions was tested by repeated measures ANOVA for comparison of data among LD, DD and LL. In cases in which ANOVA rejected the null hypothesis, the Holm–Sidak test was used for *post hoc* multiple pairwise comparisons (Ludbrook, 1998).

Raster diagrams were constructed for each animal across the 79 day study and inspected for evidence of temporal patterns in quiescent behaviour. The diagrams were constructed with modulo adjustable from 1 day to 7 days. After examination of the raster diagrams, along with frequency histograms for active bout duration, a quiescent bout 'cluster' was defined as follows: the interval extending from the start of the first quiescence bout to the end of the last quiescence bout of a sequence of three or more bouts separated by active bouts of 300 min or less. A paired *t*-test was used to compare data between cluster and inter-cluster intervals.

In each snail, we tested for serial correlations between quiescence versus prior and subsequent active intervals, on both a per-bout and per-cluster timescale. Specifically, Pearson Product Moment correlation coefficient (r) was used to test for correlations between quiescent bout duration and prior or subsequent active bout durations. This procedure was also used to test for correlations between cluster durations and inter-cluster intervals, cluster quiescence time and inter-cluster duration, and cluster quiescent bout number and inter-cluster duration.

To gain further insight into state dynamics, survival analysis was performed on quiescent bout durations and active bout durations using the Kaplan–Meier approach (Norman et al., 2006):

$$S_{t_i} = \frac{(r_i - d_i)}{r_i} S_{t_{i-1}}, \quad (1)$$

where S_{t_i} is the proportion of the original number of bouts surviving at the end of the run time bin t_i, $S_{t_{i-1}}$ is the proportion of the original number of bouts remaining one run time bin before t_i, r_i is the number of bouts remaining at the start of run time bin t_i, and d_i is the number of bouts that terminated during run time bin t_i.

Bouts immediately preceding and following periods of missing data, and quiescence bouts at the water surface were censored. Hence, the data included submerged quiescence bouts and their associated active intervals under all lighting conditions (having established that LD, DD and LL were not different – see Results). Each snail was analyzed individually to avoid inadvertent biasing of the data in favour of power law behaviour (Anderson, 2001). Graphical analysis was used to assess whether the survival curves exhibited mono-exponential, bi-exponential or power law dynamics. Thus, a mono-exponential relation is given by:

$$S_{t_i} = A\mathrm{e}^{-t/r}, \quad (2)$$

where A is a regression constant (value at time 0), t is the interval (min) since the start of a bout and τ is the exponential time constant. The mono-exponential curve yields a linear relationship when expressed in semi-logarithmic coordinates ($\ln S_{t_i}$ *vs* t), with slope $-1/\tau$.

A power law relation is given by:

$$S_{t_i} = At^{-\alpha}, \quad (3)$$

where α is the characteristic exponent of the survival curve. A power law curve yields a linear relation after double-logarithmic transformation ($\ln S_{t_i}$ *vs* $\ln t$), with slope $-\alpha$.

A bi-exponential relation is described by the sum of two exponential components:

$$S_{t_i} = A\mathrm{e}^{-t/\tau_1} + B\mathrm{e}^{-t/\tau_2}, \quad (4)$$

where A and B are regression constants, and τ_1 and τ_2 are corresponding time constants of the two exponential components. Initial estimates of these parameters were derived from semi-logarithmic least squares linear regression by the technique of exponential peeling.

As graphical analysis can be imprecise (Clauset et al., 2009), discrimination between bi-exponential and power law curves was achieved by nonlinear regression techniques using a Levenberg–Marquardt algorithm to fit the data. The regression parameter values estimated using graphical analysis were used as initial values in the nonlinear regression. Goodness of fit was estimated by the R^2 statistic and the more powerful predicted residual sum of squares (PRESS) statistic. Paired t-test was used to determine whether bi-exponential or power law regressions were a better description of the data for each of these goodness of fit statistics across the sample of eight snails.

All statistical procedures were performed using Sigmastat v.3.5 (Systat Software Inc., Point Richmond, CA, USA). In each snail, median values were taken as the measure of central tendency, as most variables exhibited a skewed distribution. A grand mean (and standard error of the mean, s.e.m.) was then calculated across animals. Statistical tests were performed on the samples of animal medians (i.e. N=8 in all tests). The null hypothesis was rejected when P<0.05.

RESULTS

Lymnaea stagnalis were found to become spontaneously quiescent at frequent but irregular intervals. Quiescent snails remained attached to the substrate. The aspect ratio of the foot decreased relative to that during locomotion, becoming less elongated both anteriorly and posteriorly, but the snails did not withdraw fully into the shell (Fig. 1). The tentacles also shortened to a variable extent (but did not withdraw completely) and appeared less turgid and sometimes curved (as shown in Fig. 1). Rasping movements of the radula were absent. When attached to a vertical surface, the shell was often observed to fall away slightly, suggesting partial relaxation of the columella muscle. Snails were not observed to assume any particular orientation or to occupy a specific site within the tank during quiescence.

Stimulus responses

Quiescent snails could be aroused in response to appetitive and aversive stimuli. Rasping motion of the radula was observed following a gentle bolus injection of sucrose solution (an appetitive stimulus) directed at the head. Quiescent snails were compared with active snails by recording latency to the first bite and number of bites taken in the first 30 s following injection. Water was used for sham stimuli. Using sucrose solution, snails responded in 124 of a total of 138 trials (90% response rate). In contrast, sham trials resulted in only five responses in a total of 84 trials (6% response rate). The following data are mean ± s.d. of N samples. Using sucrose solution, in animals that responded to the stimulus, the latency to first bite was significantly shorter (P<0.0001) in active snails (2.9±1.1 s, N=21) than in quiescent snails (20.7±13.0, N=35), and the initial bite rate (number of bites in the first 30 s) was significantly higher (P<0.0001) in active snails (10.8±2.1, N=47) than in quiescent snails (2.0±0.9, N=21; see Fig. 2A).

Responsiveness to aversive stimuli was tested by recording the number of stimuli needed to elicit a response and subsequent duration of the withdrawal reaction following a tactile stimulus to the head. The number of stimuli for quiescent snails (3.3±1.2 s, N=21) was significantly greater (P<0.0001) than that for active snails (1.4±0.5 s, N=21), and the time to re-emergence was significantly longer (P<0.0001) in quiescent snails (29.5±12.0 s, N=21) than in active snails (14.8±7.8 s, N=21). Thus, quiescent snails required more stimuli to initiate a response, but once a response was elicited it was maintained for longer than in active snails (Fig. 2B). Sham stimuli, involving approach of the probe without contact, failed to induce a withdrawal response in all of 42 trials.

Temporal organisation of quiescence

Time-lapse recordings were used to quantify the quiescent behaviour of eight snails over 79 days. Some data were lost because of technical problems, yielding a total of 73 full days of analyzed data. All quiescence start and end times were recorded to the nearest 15 s.

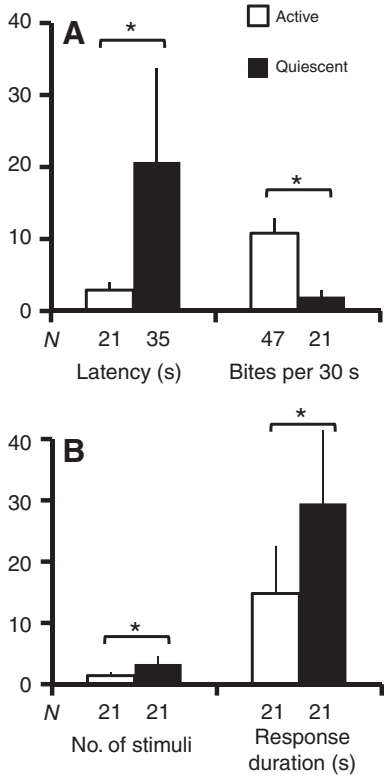

Fig. 2. Mean (+s.d.) responses to appetitive and aversive stimuli in active and quiescent snails. (A) Appetitive stimuli (bolus injection of sucrose solution to the head). Responsiveness, measured as a reduced latency to first radula rasping cycle ('bite'), was greater in active than in quiescent snails. Response intensity, measured as the initial rate of radula activity (number of 'bites' in the first 30 s), was greater in active than in quiescent snails. (B) Aversive stimuli (tactile stimulus to the head). Responsiveness, measured as the number of stimuli required to initiate a defensive withdrawal response, was higher in quiescent than in active snails (i.e. they needed more stimuli to elicit a response). Response intensity, measured as the duration of the withdrawal response, was greater in quiescent than in active snails. In all cases, separate snails were used in each trial, each snail was used only once. The number of trials (*N*) is given below each bar. **P*<0.0001.

Visual inspection of circadian raster charts (modulo 24 h) revealed little or no evidence of circadian rhythmicity in quiescence under all lighting regimes (LD16:8, DD and LL). A representative example is shown in double-plotted format in Fig. 3A. However, this subjective impression was not entirely confirmed by statistical analysis of data binned into six 4 h windows per day under LD16:8. Repeated measures ANOVA revealed a statistically significant effect of time of day on the frequency of quiescence bouts (P=0.028), with the *post hoc* Holm–Sidak multiple pairwise comparisons test indicating that quiescence bouts were less frequent during the first 4 h interval following lights on (ZT0–4) than during the early dark phase (ZT16–20) (Fig. 4A). This result was confirmed for the subset of submerged quiescent bouts (P=0.011), but not for surface bouts (P=0.309, although it should be noted that the power of the latter test was low at 0.101 owing to the relatively low sample size for surface bouts). Quiescent bout durations were not affected by time of day when data were assessed in 4 h time intervals (Fig. 4B), both for surface bouts (P=0.855) and submerged bouts (P=0.157). However, paired *t*-test revealed a small, but statistically significant, difference in submerged quiescent bout durations between light (23.3±1.2 min, N=8) and dark (26.2±1.2, N=8) phases under LD16:8 conditions (P=0.018). Surface bout durations did not differ between light and dark under LD16:8 conditions (P=0.855). The frequency of quiescence bouts did not differ between light and dark (paired *t*-test, P=0.154 and P=0.147 for surface and submerged bouts, respectively).

Further inspection of raster charts using variable modulo revealed a longer-term (infradian) pattern. A representative example is shown at modulo 7 days in Fig. 3B. In all snails, the quiescence bouts tended to be grouped into 'clusters' separated by long inter-cluster intervals containing only a few sporadic quiescence bouts. Quiescence clusters were arbitrarily defined with reference to raster charts and active bout duration frequency distribution charts (see Materials and methods), and then quantified. Quiescence clusters had a mean duration of 664±42 min (range, 204±61 to 2012±896) and contained 7.3±0.5 sleep episodes [range, 4 (defined) to 21.6±7.3]. Durations of inter-cluster intervals were 2244±238 min (range, 378±69 to 8916±2084). Repeated measures ANOVA of data grouped into six 4 h intervals across the day revealed that the frequency of onset of quiescence clusters was affected by time of day under a LD16:8 light cycle (P=0.029) (Fig. 4C). Specifically, the Holm–Sidak test revealed that quiescence clusters were less likely to start in the final 4 h interval (ZT20–24) than in the interval ZT4–8. Paired *t*-test of the data pooled into light and dark intervals confirmed that the frequency of quiescence cluster initiation was significantly lower in the dark than in the light (P=0.006). An inverse trend in the frequency of cluster termination (i.e. greater tendency to end a quiescence cluster in the dark phase) was of marginal statistical significance (paired *t*-test, light *vs* dark, P=0.051, power of test=0.445; Fig. 4C).

Over the long term, *Lymnaea* spent 9±1% of the total recording time in the quiescent state. Approximately 76% of the quiescence bouts occurred in clusters, and clusters represented approximately 25% of the total recording time. Within a cluster, 31.3±1.4% of the time was spent in quiescence, whereas the snails were quiescent for only 1.3±0.2% of the long inter-cluster intervals. The durations of quiescent and active bouts were very variable. In all variables, the within-animal variability was substantially greater than the between-animal variability, as judged by the corresponding ranges and coefficients of variation of the data.

There was a statistically significant difference between durations of surface and submerged quiescence bouts (Mann–Whitney Rank Sum Test, P<0.001). Surface bouts were 58.4±7.9 min (range, 17.7±12.2 to 155.5±65.7). This was 2.7-fold longer than the submerged bouts (21.6±1 min, range 3.9±2.0 to 438.5±60.4). However, surface quiescence occurred far less often than submerged quiescence in all snails: 177 of a total of 2358 quiescence bouts (7.2%) were at the water surface. For both surface (P=0.326) and submerged (P=0.118) quiescence there was no statistical difference between intra-cluster and inter-cluster bout durations. That is, quiescence bout duration was strongly affected by being at the water surface, but not by whether the bout was part of a cluster. The duration of active bouts (i.e. intervals between quiescence bouts) was 68.4±7.4 min (range, 4.5±0.6 to 4788±297).

Repeated measures ANOVA revealed that there were no statistically significant differences between light cycle conditions (LD16:8 *vs* DD *vs* LL) for surface and submerged quiescence and active bout durations, cluster duration, inter-cluster duration and total quiescence time within a cluster (all P>0.14). However, LL was found to induce a small decrease in the inter-cluster active bout duration [the Holm–Sidak test revealed that LL was statistically different from DD (P=0.008), whereas LD was intermediate and not significantly different from either LL or DD].

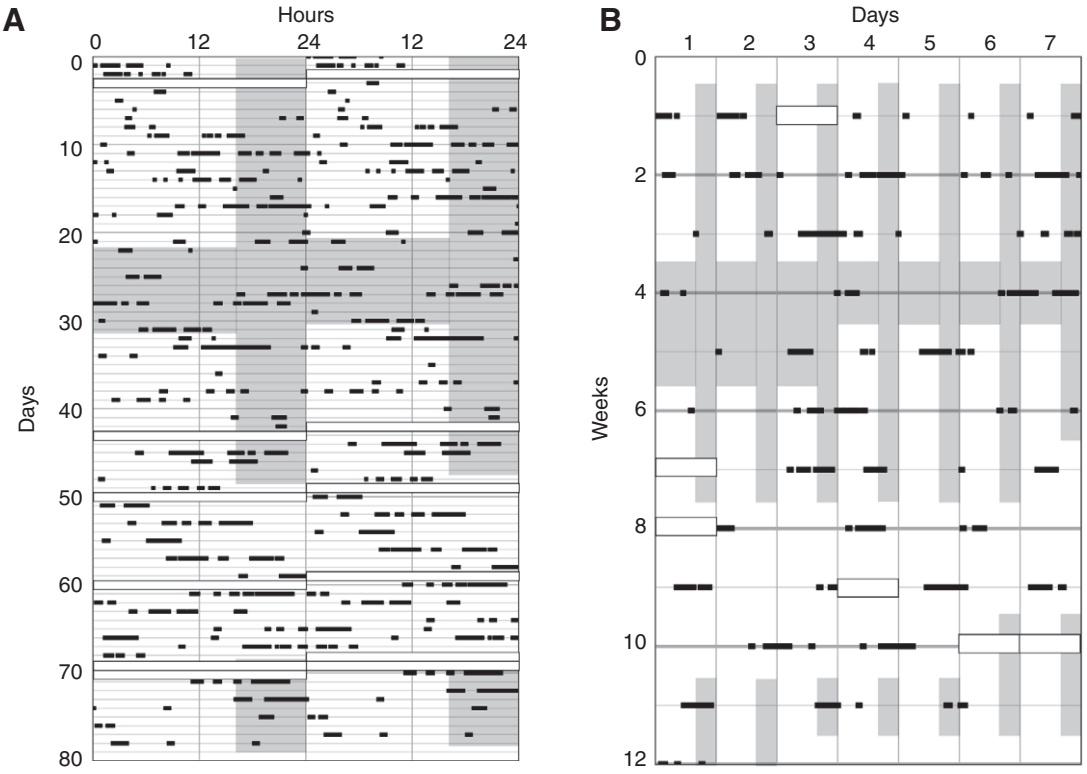

Fig. 3. Raster-style diagram illustrating the distribution of quiescence clusters over time in a representative snail. Dark bars indicate quiescence. Note that at this resolution some short active bouts are not visible. White background indicates lights on, light grey background indicates darkness. Blank bars indicate missing data. (A) Modulo 24 h. Time of day [Zeitgeber time, where ZT0 is 05:00 h Eastern Standard Time (EST), time of lights on during LD16:8 intervals] is shown on the abscissa and days of study on the ordinate. These data are drawn double-plotted, such that each day is shown both to the right of, and below, each preceding day. Note the absence of any obvious circadian periodicity in the data. (B) Modulo 7 days. Days of the week are shown on the abscissa and weeks of study on the ordinate; data are shown in single-plot format. Note that quiescence is consolidated into clusters that are organized on an infradian timescale. Relatively few sporadic quiescent bouts occur between clusters. Snails sometimes remained active for intervals exceeding 2 days.

Given the above results, survival analysis was performed on quiescence bout durations and active bout durations using submerged bouts over the entire 79 day study (i.e. data from different lighting conditions were pooled). Fig. 5B shows that quiescence bout durations were described by a mono-exponential function, above a minimum bout duration of 5–10 min. Across the eight snails, the mean (±s.e.m.) time constant was 15.5±1.1 min (R^2=0.995±0.001). The insets in Fig. 5B illustrate that a semi-logarithmic plot of the data is linear, whereas a double logarithmic plot is curved, excluding a power law distribution.

The survival curve for the active bout duration data is shown in Fig. 5A. The semi-logarithmic plot was linear in the tail of the distribution but curved for quiescence bouts of less than 218±19 min, thus excluding a simple mono-exponential model for active bouts. The double-logarithmic plot contained a linear segment bounded by both lower (19±1 min) and upper (499±13 min) limits. Graphical analysis by the technique of 'exponential peeling' was used to show that a bi-exponential function is a plausible description of the active bout duration survival curve over the range 19±1 to 1489±52 min (Fig. 5A). Using nonlinear regression, it was confirmed that a bi-exponential fit (R^2=0.999±0.0003, PRESS=0.0049±0.0008) was superior (P<0.001) to a power law fit (R^2=0.970±0.003, PRESS=0.0884±0.0112). These nonlinear regressions were performed using the 'optimal' segments of the data for each (i.e. within the limits indicated above for the linear segments of the logarithmic plots). It can be seen in Fig. 5A that the bi-exponential function describes a larger proportion of the distribution (73±2%) than the power law function (65±2%). The time constant of the slowly decaying exponential component (τ_2=592±48 min) was approximately an order of magnitude greater than that of the rapidly decaying component (τ_1=62±4 min).

There were no significant correlations between quiescence bout duration and prior or subsequent active bout durations. Likewise, there were no significant correlations between inter-cluster interval and cluster duration, cluster quiescence time or cluster quiescence bout number. Quiescence was found to vary substantially across the 79 day study and the variability was aperiodic, resembling a random walk (Fig. 6).

DISCUSSION

This study has found that the great pond snail, *Lymnaea stagnalis*, exhibits an intermittent quiescent behaviour that has some, but perhaps not all, of the characteristics of sleep. The key behavioural criteria – motor suppression (which manifests as behavioural quiescence) and reduced sensory-motor responsiveness, both of which are rapidly reversible, either spontaneously or in response to moderate natural stimuli (Hendricks et al., 2000b) – were fulfilled in quiescent *Lymnaea*. We therefore conclude that quiescence in the snail is a sleep-like state.

These data contribute to the growing list of invertebrate species that have been shown to express a sleep-like state, and add further support to the suggestion that sleep is an ancient evolutionary

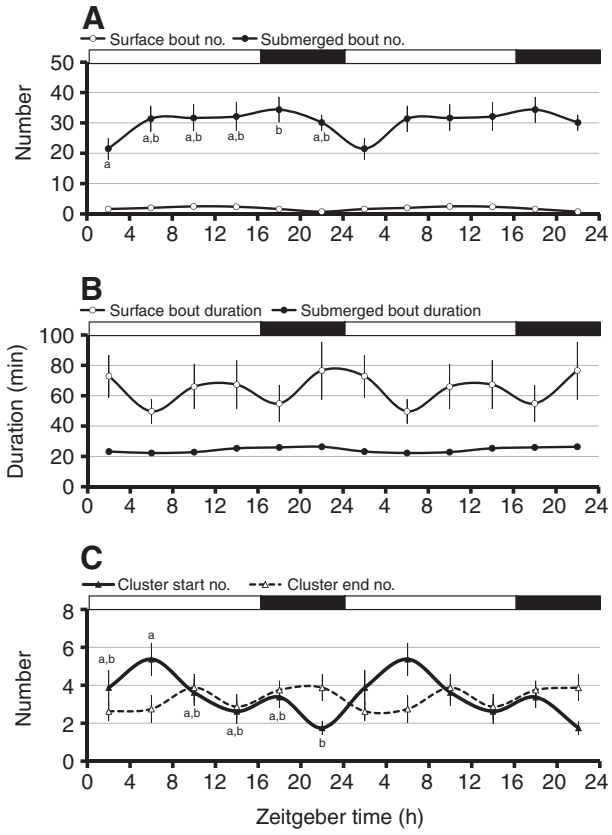

Fig. 4. Effect of time of day (Zeitgeber time, where ZT0 is 05:00 h EST, time of lights on) on quiescence behaviour during 45 days under a LD16:8 light cycle. Data were pooled into six daily 4 h time bins. Bars above each panel illustrate the light (white bar) and dark (black bar) phases of the day. Mean (±s.e.m.) data for eight snails are shown. In some cases error bars are within the boundary of the symbol. For visual clarity, the data are shown double-plotted. (A) Total number of quiescent bouts initiated in each time bin over the 45 days of recording. Note that submerged quiescent bouts (black) were much more frequent that surface bouts (white). Submerged quiescent bouts were least numerous in the first 4 h of the day. (B) Durations of submerged (black) and surface (white) quiescent bouts. There was no effect of time of day on bout durations. (C) Total number of quiescent bout clusters that began (black symbols, solid line) and ended (white symbols, dashed line) in each 4 h time bin across the 45 days of recording. Clusters were least likely to begin at night (ZT20–24) and most likely to begin during the morning (ZT4–8). An increased tendency for clusters to end at night was of marginal statistical significance. In A and C, points with shared letters (a,b) are statistically similar.

adaptation (Piscopo, 2009). The vast majority of invertebrate species that have been shown to express a sleep-like state belong to the phylum *Arthropoda*, which are relatively complex (anatomically, neurologically and behaviourally) compared with most other invertebrates. Compelling evidence for a sleep-like state has been published for the honey bee (*Apis melifera*) (Kaiser, 1988; Kaiser and Steiner-Kaiser, 1983; Sauer et al., 2004; Sauer et al., 2003), cockroach (*Blaberus giganteus*) (Tobler and Neuner-Jehle, 1992), fruit fly (*Drosophila melanogaster*) (Hendricks et al., 2000a; Shaw et al., 2000), scorpion (*Heterometrus longimanus*) (Tobler and Stalder, 1988) and crayfish (*Procambarus clarkii*) (Mendoza-Angeles et al., 2007; Mendoza-Angeles et al., 2010; Ramon et al., 2004). Other than the arthropods, only the octopus (Brown et al., 2006), a relatively complex cephalopod mollusc, and a nematode (*Caenorhabditis elegans*) (Raizen et al., 2008) have been studied in detail and found to exhibit behavioural characteristics that are sleep-like. For many reasons, *C. elegans* has tremendous potential as a model organism for sleep studies. However, the sleep-like state (known as 'lethargus') is restricted to only four specific stages in the early life cycle of this species and is absent in the adult, raising questions as to the extent to which lethargus is truly homologous with sleep. Likewise, *Octopus* is perhaps not an optimal candidate for a simple animal model of sleep because it is one of the most complex of invertebrates. Thus, *L. stagnalis* may represent a very good candidate because, as we have shown here, it expresses a sleep-like state in adulthood, yet is simple enough to facilitate neurophysiological experimentation at the cellular level (Mills and Winlow, 1979; Lukowiak et al., 2003). Its potential value as a simple animal model of sleep is enhanced by recent and ongoing genome analysis in this species (Feng et al., 2009).

The cessation of locomotor activity and rasping movements of the radula, the partial retraction of the tentacles and the reduction in the aspect ratio of the foot all suggest that quiescence involves a general suppression of motor output in *Lymnaea*. This state of motor quiescence was rapidly reversed either spontaneously or by the presentation of an appetitive stimulus (chemical stimulation by sucrose solution, which stimulates feeding behaviour) or an aversive stimulus (tactile stimulation of the head, which stimulates a transient withdrawal response followed by locomotion). The responsiveness of the snails to both of these stimuli was significantly reduced when the stimuli were presented during the putative sleep-like quiescent state compared with during active locomotion. Similar reductions in sensory responsiveness, measured either behaviourally or electrophysiologically, have been reported in other invertebrates during sleep-like quiescent states, including *Octopus* (Brown et al., 2006), *Apis* (Kaiser and Steiner-Kaiser, 1983), *Drosophila* (Hendricks et al., 2000a; Shaw et al., 2000), *Blaberus* (Tobler and Neuner-Jehle, 1992), *Heterometrus* (Tobler and Stalder, 1988), *Procambarus* (Ramon et al., 2004) and *Caenorhabditis* (Raizen et al., 2008). Fig. 2A shows that responsiveness to sucrose solution in active snails was approximately five times greater than that in quiescent snails, as judged by latency to first bite (radula rasping motion) and initial bite rate. These data concur with an earlier study in which feeding responses to sucrose were shown to be inversely related to a 'behavioural state score', where low scores corresponded to low general activity levels (Tuersley and McCrohan, 1987). Similarly, in the present study the responsiveness to tactile stimuli was approximately half as great in quiescent snails as in active snails, as indicated by the increased number of stimuli required to elicit a withdrawal response (Fig. 2B). It was interesting to note that the ensuing defensive response was more intense in quiescent snails, as judged by an increased time to re-emergence. One possible explanation for this is that stimulated arousal may activate a stress response that potentiates the withdrawal reaction. In mammals, arousal from sleep is known to induce transient cardio-respiratory activation and an autonomic stress reaction following spontaneous and stimulated awakening (Horner, 1996).

Other criteria that are often used to define a resting state as 'sleep-like' include the adoption of a stereotypical resting posture in a preferred location. Quiescence in *Lymnaea* was defined as a posture consisting of a shortening of the foot, partial relaxation of the tentacles and an absence of radula activity. Other behaviours featuring periods of inactivity were excluded from this definition for reasons given in Materials and methods. Quiescent snails were always attached to a solid substrate and most (>90%) quiescent bouts occurred with the animals submerged and attached to the vertical walls of the aquarium. However, this does not necessarily imply

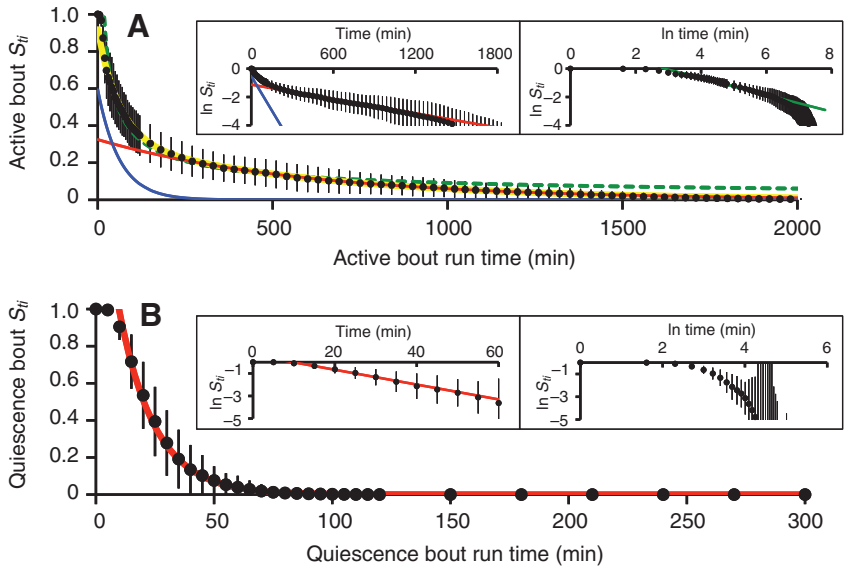

Fig. 5. Kaplan–Meier survival curve analysis of durations of active bouts and submerged quiescent bouts. In both panels, black circular symbols indicate mean (±s.e.m.) data for eight snails. (A) Bi-exponential curve (yellow) was found to provide the best fit to active bout survival fraction (S_{ti}) by nonlinear regression (Levenberg–Marquardt algorithm). The two component exponential curves, calculated from regression parameters, are shown in red and blue. A semi-logarithmic plot of the data (left inset) indicates a linear tail (red regression line) plus the rapidly decaying component (blue) derived by the technique of exponential peeling. A power law curve (green dashed line) yielded an inferior fit to the data, despite presenting a relatively linear curve on double-logarithmic coordinates (right inset, green regression line). (B) Exponential curve (red) fitted to quiescent bout survival fraction. The semi-logarithmic plot (left inset) was linear, whereas a double-logarithmic plot (right inset) was curved thereby excluding a power law fit. Note that all quiescent bouts lasted at least 5–10 min, then probability of arousal remained constant thereafter.

that these animals have a preference for submerged vertical attachment sites and may instead simply reflect the fact that the area of the submerged vertical walls of the observation chambers was approximately 90% of the total solid area. Hence, it appears that quiescence was distributed randomly within the tank. Although it is possible that this may have been influenced by the relatively 'impoverished' environment in the observation tank, informal observation of animals in the more complex habitat of the stock tank (gravel substrate, opportunities for shelter, regions of increased water turbulence and the presence of other snails) also failed to identify a preferred site for quiescence. It was of interest to note that quiescence bouts at the water surface were, on average, over twice the duration of submerged quiescence. It is possible that the surface bouts may have consisted of two or more bouts separated by brief 'arousals' containing undetected breathing cycles. Alternatively, quiescence bouts may have been extended by the relative hyperoxia that they experience at the air-water interface.

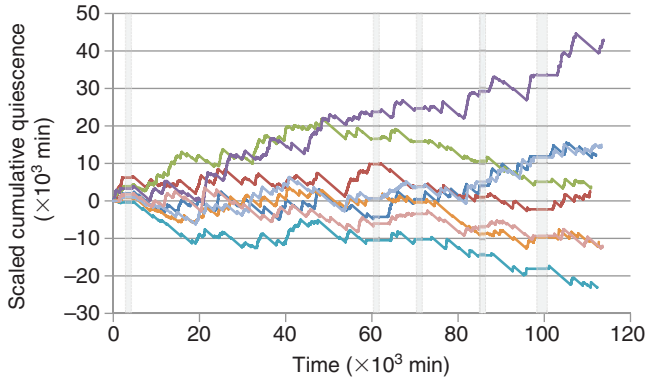

Fig. 6. Cumulative expression of quiescence in eight snails over a 79 day observation period. Grey bars indicate missing data. For clarity of presentation, data were scaled according to the sample mean active time/quiescent time ratio. Thus, each minute of quiescence was assigned +11 units and each minute of activity was assigned –1 unit. The data illustrate that expression of quiescence resembles a Markovian random walk, as predicted by the exponential probability distributions of state durations.

This issue deserves further attention because it raises the interesting possibility that quiescence bout duration may be influenced by a respiratory chemoreflex in these animals.

In mammals, extended wakefulness is followed by a 'sleep rebound' usually featuring increased sleepiness, reduced sleep latency, increased sleep consolidation, increased sleep time and, perhaps, increased sleep 'intensity'. This response has been interpreted as being evidence for the homeostatic regulation of sleep (Borbely and Achermann, 1999). The timing (and perhaps also the quantity) of sleep is also regulated by the circadian timing system in mammals (Franken and Dijk, 2009). However, neither of these mechanisms featured prominently in the regulation of quiescence in *Lymnaea*.

The temporal dynamics of quiescence and activity were best characterised as a stochastic process. Survival analysis revealed that submerged quiescence bout durations were described by an exponential probability distribution (Fig. 5), implying that the likelihood of state transition is 'memory-free' (dependent only on its current state). The analysis revealed that after the first 5–10 min of quiescence the probability of arousal remained constant; approximately a 30% chance of 'awakening' in any given 5 min interval. The median duration of quiescence bouts was unaffected by whether they occurred sporadically or as part of a cluster, indicating that the clustering of the behaviour arose through the modulation of activity and not quiescence. Thus, in *Lymnaea*, the duration of active bouts was described by the sum of two exponential functions, tentatively suggesting that two processes may be involved in the regulation of active bout maintenance and/or quiescence onset. The adaptive advantages of the 'consolidation' of quiescence into clusters are unclear and the underlying mechanisms are unknown. Both of these questions represent opportunities for further investigation.

There was no correlation between duration of activity and duration of subsequent quiescence, either for individual bouts or for bout clusters, a surprising finding given the very large (>1000-fold) range of spontaneous active bout durations. These observations – stochastic state transitions and the absence of serial correlations between quiescence and activity – suggest that a homeostatic drive, if it exists, does not operate over the bout-to-bout or cluster-to-cluster time scales in *Lymnaea*, and therefore may not contribute to the

baseline regulation of sleep-like quiescence in this species. Thus, the 'hypnogram' of *Lymnaea* can be viewed as a Markovian random walk (Fig. 6). Such a mechanism will tend to maintain adequate long-term sleep-like quiescence given appropriate time constants of the process, and may be all that is required for sleep homeostasis in an organism in which the 'need' for sleep is low, or the tolerance for short-term variation in sleep is high. It is worth noting here that human nocturnal sleep dynamics have been successfully modelled by Markov analysis (Kim et al., 2009), and similar short-term sleep–wake dynamics have been described in other mammals (Lo et al., 2004), suggesting that there may be a class of mechanism (a 'stochastic oscillator') at the core of sleep regulation that is conserved across phylogeny. Analyses of short-term sleep–wake dynamics in other invertebrate and vertebrate species would be of interest as they may yield insight into the evolution of behavioural state regulation.

Although it can be argued that a Markovian mechanism may, at least in principle, suffice for the maintenance of adequate long-term quiescence, the data presented in this study cannot exclude the possibility that a 'quiescence rebound' may occur in *Lymnaea* if active bouts exceed a minimum duration. Maximum spontaneous active bout durations were in excess of 3 days in the present study, suggesting that the putative threshold for activation of a quiescence rebound may be at least this long. Unfortunately, attempts to test this hypothesis by imposing quiescence deprivation using three mechanical methods and one chemical approach were unsuccessful. Repetitive exposure of the snails to the air using a 'tidal' mechanism (slow cyclic tank drainage and refilling) caused the animals to track the water surface initially, but proved to be detrimental to the health of the snails, which either became torpid or died after a day or two. Likewise, agitation of snails attached to lettuce leaf at the water surface using an orbital shaker was effective for about a day before snails began to die. A milder stimulus of intermittent agitation using bursts of bubbles was found to be ineffective in preventing quiescence. Finally, placing the animals in an aquarium containing water obtained from a tank housing crayfish, a natural predator of *Lymnaea* (Orr et al., 2007), caused an increased incidence of apparent torpor, often in association with crawling clear of the water. Hence, this study remains inconclusive regarding whether *Lymnaea* possess a typical homeostatic response and resolution of this issue must await the development of a benign stimulus that effectively prevents quiescence for at least several days. Sleep rebounds are highly variable in vertebrates and invertebrates, rarely meeting the homeostatic requirement of full compensation for sleep loss, and several instances of little or no response have been reported. Examples of the latter include male *Drosophila* (CS strain) after 6 h of mechanical stimulation (Huber et al., 2004), zebrafish (*Danio rerio*) after 6 h or 3 days of light-induced vigilance (Yokogawa et al., 2007), pigeons (*Columba livia*) following light-induced sleep deprivation (Tobler and Borbely, 1988; Berger and Phillips, 1994), golden-mantled ground squirrels (*Spermophilus lateralis*) following 3 h of gentle handling during the post-hibernation period (Larkin and Heller, 1998), squirrel monkeys (*Saimiri sciureus*) following up to 36 h of mild disturbance (Klerman et al., 1999), bottlenose dolphins (*Tursiops truncates*) following up to 5 days of auditory vigilance (Ridgway et al., 2006), and rats (*Rattus norvegicus*), which lack a NREM sleep rebound following 4 days of sleep deprivation (Rechtschaffen et al., 1999). Regardless of whether these exceptions are interpreted as evidence against a sleep homeostatic mechanism (Siegel, 2009) or as evidence of a poorly designed study (Cirelli and Tononi, 2008), in no case did the authors of those papers conclude that the animals do not sleep at all; the existence of 'sleep' as a state is not dependent on the demonstration of a particular kind of regulatory mechanism.

Time of day had only a small influence on quiescence behaviour, with slightly longer quiescent bout durations at night and reduced frequency of quiescence onset immediately after lights on (i.e. at 'dawn'). Furthermore, quiescent bout clusters were less likely to start and (perhaps) more likely to end at night. Whether this was a true circadian timing effect, or a masking effect of the light–dark cycle could not be determined because under free-running conditions (LL and DD) there was no reliable circadian phase marker in the data with which to define circadian time. There was little indication that either constant light or constant darkness had any substantial effect on quiescence, despite the presence of ocular and extra-ocular photoreceptors in *Lymnaea* that influence active behaviours (Chono et al., 2002; Stoll, 1973). Absence of circadian modulation of the activity–rest cycle (and also of stimulus response thresholds) has been reported before in this species (Tuersley and McCrohan, 1987). This contrasts with *Aplysia* (Fernandez et al., 2003; Strumwasser, 1971; Susswein et al., 1983; Ziv et al., 1991) and *Octopus* (Brown et al., 2006; Cobb et al., 1995), both of which have a strong circadian variation in quiescence behaviour. However, there is no reason to suppose that circadian rhythmicity is a necessary characteristic of sleep, as some mammalian species, most notably the guinea pig, *Cavia porcellus* (Tobler and Franken, 1993), have little or no circadian sleep-wake rhythms, and others have been shown to express normal sleep after surgical (Mistlberger et al., 1983), photic (Eastman and Rechtschaffen, 1983; Larkin et al., 2004) and genetic (Shiromani et al., 2004) ablation of circadian rhythms.

In conclusion, we have identified a spontaneously occurring quiescent behaviour in *L. stagnalis* that satisfies many of the generally accepted behavioural criteria for sleep. Most importantly, it features postural immobility that can be rapidly aroused both spontaneously and in response to moderate stimuli. The responsiveness of the animals to sensory stimulation is significantly lower in this putative sleep-like state than when the snails are active. However, quiescence in *Lymnaea* differs from sleep in mammals and sleep-like rest in arthropods in that we failed to obtain evidence for a rebound mechanism in homeostatic regulation of the state. Instead, the state appeared to be regulated by stochastic mechanisms in which both quiescence and activity had defined probabilities of duration. Furthermore, there was only weak evidence for a role of circadian rhythms in the regulation of the quiescent behaviour, which was organized into clusters and inter-cluster intervals spanning an infradian time scale. However, we argue that rebound mechanisms and circadian rhythms are not necessary for the occurrence of sleep, but merely serve to modulate the expression of the state. We suggest that *L. stagnalis*, by virtue of its anatomical simplicity and neurophysiological tractability (Mills and Winlow, 1979; Lukowiak et al., 2003), may prove useful in the investigation of cellular mechanisms of sleep regulation and sleep function.

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